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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/19, C07K 14/52, C12Q 1/68, C12N 15/11, C07K 16/24, G01N 33/68, C12N 1/21

A1

(11) International Publication Number: WO 96/32481

(43) International Publication Date: 17 October 1996 (17.10.96)

(21) International Application Number: PCT/US96/05102

(22) International Filing Date: 12 April 1996 (12.04.96)

(30) Priority Data:
08/421,144 13 April 1995 (13.04.95) US

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(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: NEW CHEMOKINE EXPRESSED IN EOSINOPHILS

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                 9          18         27         36         45         54
5' ATG AAG GTC TCC GTG GCT GCC CTC TCC TGC CTC ATG CTT GTT ACT GCC CTT GGA
   Met Lys Val Ser Val Ala Ala Leu Ser Cys Leu Met Leu Val Thr Ala Leu Gly

                63         72         81         90         99        108
   TCC CAG GCC CGG GTC ACA AAA GAT GCA GAG ACA GAG TTC ATG ATG TCA AAG CTT
   Ser Gln Ala Arg Val Thr Lys Asp Ala Glu Thr Glu Phe Met Met Ser Lys Leu

               117        126        135        144        153        162
   CCA TTG GAA AAT CCA GTA CTT CTG GAC ATG CTC TGG AGG AGA AAG ATT GGT CCT
   Pro Leu Glu Asn Pro Val Leu Leu Asp Met Leu Trp Arg Arg Lys Ile Gly Pro

               171        180        189        198        207        216
   CAG ATG ACC CTT TCT CAT GCT GCA GGA TTC CAC GCT ACT AGT GCT GAC TGC TGC
   Gln Met Thr Leu Ser His Ala Ala Gly Phe His Ala Thr Ser Ala Asp Cys Cys

               225        234        243        252        261        270
   ATC TCC TAC ACC CCA CGA AGC ATC CCG TGT TCA CTC CTG GAG AGT TAC TTT GAA
   Ile Ser Tyr Thr Pro Arg Ser Ile Pro Cys Ser Leu Leu Glu Ser Tyr Phe Glu

               279        288        297        306        315        324
   ACG AAC AGC GAG TGC TCC AAG CCG GGT GTC ATC TTC CTC ACC AAG AAG GGC CGA
   Thr Asn Ser Glu Cys Ser Lys Pro Gly Val Ile Phe Leu Thr Lys Lys Gly Arg

               333        342        351        360        369        378
   CGT TTC TGT GCC AAC CCC AGT GAT AAG CAA GTT CAG GTT TGC ATG AGA ATG CTG
   Arg Phe Cys Ala Asn Pro Ser Asp Lys Gln Val Gln Val Cys Met Arg Met Leu

               387        396        405
   AAG CTG GAC ACA CGG ATC AAG ACC AGG AAG AAT  3'
   Lys Leu Asp Thr Arg Ile Lys Thr Arg Lys Asn
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(57) Abstract

The present invention relates to novel nucleotide and amino acid sequences for a novel C-C chemokine initially found in a cDNA library from blood cells of an individual having Hypereosinophilic Syndrome. The present invention also provides for antisense molecules to the nucleotide sequences which encode EEC, expression vectors for the production of purified EEC, antibodies capable of binding specifically to EEC, hybridization probes or oligonucleotides for the detection of EEC-encoding nucleotide sequences EEC, diagnostic tests for chemokine activation based on EEC-encoding nucleic acid molecules and antibodies capable of binding specifically to EEC.

NEW CHEMOKINE EXPRESSED IN EOSINOPHILS

TECHNICAL BACKGROUND

The present invention relates to novel nucleotide and amino acid sequences of a chemokine found in eosinophils and to the use of these sequences in the diagnosis and treatment of disease.

BACKGROUND ART

Chemokines

The chemokines are a family of cytokines that are produced when the immune system responds to non-self antigens, such as invading microorganisms or antigens of an incompatible tissue type and are associated with leukocyte trafficking in abnormal, inflammatory or diseased conditions. Chemokines mediate the expression of particular adhesion molecules on endothelial cells and they generate gradients of chemoattractant factors which activate specific cell types. In addition, the chemokines stimulate the proliferation of specific cell types and regulate the activation of cells which bear specific receptors. These activities demonstrate a high degree of target cell specificity.

The chemokines are small polypeptides, generally about 70-100 amino acids in length, 8-11 kD in molecular weight and active over a 1-100 ng/ml concentration range. Initially, they were isolated and purified from inflamed tissues and characterized relative to their bioactivity. More recently, chemokines have been discovered through molecular cloning techniques and characterized by structural as well as functional analysis.

The chemokines are related through a four-cysteine motif which is based primarily on the spacing of the first two cysteine residues in the mature molecule. Currently the chemokines are assigned to one of two families, the C-C chemokines (α) and the C-X-C chemokines (ß). Although exceptions exist, the C-X-C chemokines generally activate neutrophils and fibroblasts while the C-C chemokines act on a more diverse group of target cells which include monocytes/macrophages, basophils, eosinophils, T lymphocytes and others. The known chemokines of both families are synthesized by many diverse cell types as reviewed in Thomson A. (1994) The Cytokine Handbook, 2d Ed. Academic Press, NY. The two groups of chemokines will be described in turn.

C-C chemokines appear to have less N-terminal processing than the C-X-C chemokines. Known human and/or murine C-C chemokines include MIP-1α and ß; I-309; RANTES and MCP-1. The macrophage inflammatory proteins alpha and beta (MIP-1α and ß) were first purified from stimulated mouse macrophage cell line

and elicited an inflammatory response when injected into normal tissues. At least three distinct and non-allelic genes encode human MIP-1α, and seven distinct genes encode MIP-1ß.

MIP-1α and MIP-1ß consist of 68-69 amino acids which are about 70% identical in their acidic, mature secreted forms. They are both expressed in stimulated T cells, B cells and monocytes in response to mitogens, anti-CD3 and endotoxin, and both polypeptides bind heparin. While both molecules stimulate monocytes, MIP-1α chemoattracts the CD-8 subset of T lymphocytes and eosinophils, while MIP-1ß chemoattracts the CD-4 subset of T lymphocytes. In mouse, these proteins are known to stimulate myelopoiesis.

I-309 was cloned from a human γ-δ T cell line and shows 42% amino acid identity to T cell activation gene 3 (TCA3) cloned from mouse. There is considerable nucleotide homology between the 5' flanking regions of these two proteins, and they share an extra pair of cysteine residues not found in other chemokines. Such similarities suggest I-309 and TCA3 are species homologs which have diverged over time in both sequence and function.

RANTES is another C-C chemokine which is expressed in T cells (but not B cells), in platelets, in some tumor cell lines, and in stimulated rheumatoid synovial fibroblasts. In the latter, it is regulated by interleukins-1 and -4, transforming nerve factor and interferon-γ. The cDNA cloned from T cells encodes a basic 8 kD protein which lacks N-linked glycosylation and is able to affect lymphocytes, monocytes, basophils and eosinophils. The expression of RANTES mRNA is substantially reduced following T cell stimulation.

Monocyte chemotactic protein (MCP-1) is a 76 amino acid protein which appears to be expressed in almost all cells and tissues upon stimulation by a variety of agents. The targets of MCP-1, however, are limited to monocytes and basophils in which it induces a MCP-1 receptor:G protein-linked calcium flux (Charo I, personal communication). Two other related proteins (MCP-2 and MCP-3) were purified from a human osteosarcoma cell line. MCP-2 and MCP-3 have 62% and 73% amino acid identity, respectively, with MCP-1 and share its chemoattractant specificity for monocytes.

International Publication Number WO 95/17092, published June 29, 1995, and its priority document, United States Application Serial Number 08/208,339 filed March 8, 1994 disclose the nucleotide and amino acid sequence of MIP3, a chemokine found in an aortic endothelium cDNA library that has 66% similarity to MIP-1α.

The chemokine molecules have been reviewed in Schall TJ (1994)

Chemotactic Cytokines: Targets for Therapeutic Development. International Business Communications, Southborough, MA, pp 180-270; and in Paul WE (1993) Fundamental Immunology, Raven Press, New York City (NYC), pp 822-826.

Eosinophils

Eosinophils are bi- or multi-nucleate white blood cells which contain basophilic or eosinophilic granules formed during their development by highly active golgi and ribosomal machinery. The plasma membrane is not structurally distinct from that of other leukocytes, but it is characterized by immunoglobulin (Ig) receptors, particularly IgG and IgE. These cells are formed throughout life from pluripotent stem cells and play a crucial role in systemic defense protecting the body from microorganisms and foreign proteins. In comparison to a total of 7000 white blood cells per microliter of blood, the number of eosinophils is normally about 160 cells per microliter. Eosinophils, generally six days worth, are formed and stored in the bone marrow until they are recruited to the site of inflammation or invasion.

Eosinophils have a special function in parasitic infections. They attach to parasitic larvae, presumably via their Ig receptors, and undergo degranulation in response to interleukin-5 (IL-5), IL-3, granulocyte/monocyte cell stimulating factor (GM-CSF) produced by activated T cells and mast cells of the host (Abu-Ghazaleh RI, Kita H, Gleich GJ (1992) Immunol Ser 57:137-67) or other factors produced by the parasite. Degranulation releases many active species including the following: 1)hydrolytic enzymes such as peroxidase, acid phosphatase, phospholipase, B glucuronidase, ribonuclease, arylsulfatase and cathepsin; 2) highly reactive superoxides; and 3) major basic protein (MBP), an arginine-rich potent larvicidal polypeptide and eosinophil cationic protein (Capron M (1992) Mem Inst Oswaldo Cruz 87(S5):89). Eosinophils are produced in great quantities in persons with helminth infections such as hookworm, schistosomiasis, toxocariasis, trichuriasis, filariasis, strongyloidiasis, echinococcosis, cysticercosis, and trichinosis for example.

Large numbers of eosinophils also collect in tissues such as the heart, lungs, central nervous system, sinuses and skin where allergic reactions commonly occur. They are chemoattracted to the site of inflammation or invasion by eosinophil chemotactic factor, platelet activation factor, complement 5a, or IL-5 which are released by mast cells and basophils during the allergic reaction. Eosinophils neutralize slow reacting substance of anaphylaxis (a mixture of leukotrienes) and histamine released by mast cells

and basophils; produce eosinophil derived innibitor which prevents degranulation of mast cells; and phagocytize antigen-antibody complexes, all of which downregulate the hypersensitivity response.

Eosinophilia, an excess of eosinophils, i.e.more than 500 per microliter of blood, is commonly observed in patients with allergies, hay fever, asthma and reactions to drugs as common as aspirin, sulfonamides and penicillins. Eosinophilia is also associated with rheumatoid arthritis and cancers such as Hodgkins lymphoma, chronic myelogenous leukemia, and carcinomas of the lung, stomach, pancreas, ovaries, uterus and liver . Eosinophilia may cause tissue damage by excessive degranulation and is usually treated with glucocorticoid chemotherapy.

Eosinophils, their morphology, function and relation to disease are reviewed, inter alia, in Guyton AC (1991) Textbook of Medical Physiology, WB Saunders Co, Philadelphia PA; Isselbacher KJ et al (1994) Harrison's Principles of Internal Medicine, McGraw-Hill, New York City, pp. 1437-1504; and Zucker-Franklin D et al (1988) Atlas of Blood Cells, Function and Pathology, Lea and Febiger, Philadelphia PA.

Current techniques for diagnosis of abnormalities in inflamed or diseased tissues mainly rely on observation of clinical symptoms or serological analyses of body tissues or fluids for hormones, polypeptides or various metabolites. Patients often manifest no clinical symptoms at early stages of disease development. Furthermore, serological analyses do not always differentiate between invasive diseases and genetic syndromes which have overlapping or very similar ranges. Current methods of treating inflammatory conditions involve administration of steroids and other drugs with multiple side effects. The discovery of novel chemokines involved in inflammatory conditions provides the basis for the development of safer and more accurate diagnostic and therapeutic compositions and methods.

DISCLOSURE OF THE INVENTION

The present invention relates to novel nucleotide and amino acid sequences for a chemokine initially found in a cDNA library made from blood cells from a patient diagnosed with Hypereosinophilic Syndrome at the Mayo Clinic. The new gene, which is known as eosinophilic expressed chemokine, or eec (Incyte Clone 288236), encodes the polypeptide designated EEC, a new member of the C-C chemokine family. The present invention relates to the use of the nucleotide and amino acid sequences of EEC in the study, diagnosis and treatment of disease states related to leukocyte trafficking in abnormal,

inflammatory or diseased conditions, such as in Eosinophilia. Eosinophilia is defined as a dramatic increase in the number of eosinophils per microliter of blood. It has been observed in the following conditions: collagen vascular diseases such as rheumatoid arthritis, eosinophilic fasciitis, allergic angitis, periarteritis nodosa, and granulomatosis; malignancies such as Hodgkin's lymphoma, mycosis fungoides, chronic myelogenous leukemia and cancer of the lung, stomach, pancreas, ovaries or uterus; helminthic infections such as hookworm, schistosomiasis, toxocariasis, trichuriasis, filariasis, strongyloidiasis, echinococcosis, cysticercosis, and trichinosis; hypereosinophilic syndromes such as Loeffler's syndrome, Loeffler's endocarditis, eosinophilic leukemia, eosinophil myalgia, and idiopathic hypereosinophilic syndrome; and allergies and asthma.

The present invention is based in part on the amino acid homology that EEC shares with other members of the C-C chemokine family and in part upon the presence of nucleotide sequences for EEC in an eosinophilic cDNA library. The nucleotide and amino acid sequences for EEC have similarity to the nucleotide and amino acid sequences for MIP3 disclosed in International Publication Number WO 95/17092, published June 29, 1995, and its priority document, United States Application Serial Number 08/208,339 filed March 8, 1994.

The present invention is therefore based on the discovery of a novel C-C chemokine, EEC, that is associated with leukocyte trafficking in abnormal, inflammatory or diseased conditions. EEC and nucleotide sequences that encode it and oligonucleotides, peptide nucleic acid (PNA), fragments, portions or antisense molecules thereof, provide the basis for diagnostic methods for the early and accurate detection and/or quantitation of EEC associated with inflammatory or diseased conditions. For example, the eec nucleotide sequences disclosed herein, which encode EEC, or fragments thereof, may be used in hybridization assays of biopsied cells or tissues or bodily fluids to diagnose abnormalities in individuals having or at risk for inflammation.

An abnormal level of nucleotide sequences encoding EEC in a biological sample may reflect a chromosomal aberration, such as a nucleic acid deletion or mutation. Accordingly, nucleotide sequences encoding EEC provide the basis for probes which can be used diagnostically to detect chromosomal aberrations such as deletions, mutations or chromosomal translocations in the gene encoding EEC. Eec gene expression may be altered in such disease states or there may be a chromosomal aberration present in the region of the gene encoding EEC.

The present invention also provides for eec antisense molecules or EEC antagonists which may be used to block the activity of EEC, i.e., leukocyte trafficking, in conditions where it is desirable to block the activity of the chemokine, such as inflammation. Alternatively, eec sense molecules or EEC agonists may be used to enhance the activity of the EEC in conditions where it is desirable to enhance leukocyte trafficking, such as in acute or chronic infection, where it may be desirable to increase leukocyte trafficking.

The present invention also relates to expression vectors and genetically engineered host cells comprising eec nucleotide sequences for the in vitro or in vivo production of the nucleotide and amino acid sequences.

Additionally, the present invention relates to the use of a EEC polypeptide, or fragment or variant thereof, to produce anti-EEC antibodies and to screen for antagonists or inhibitors of the EEC polypeptide which can be used diagnostically to detect and quantitate EEC protein levels in disease states.

The present invention also relates to pharmaceutical compositions comprising effective amounts of inhibitors or antagonists of EEC protein or EEC anti-sense nucleic acid in conditions where it is desirable to reduce the activity of the chemokine, for example, in the treatment of inflammation. The present invention also relates to pharmaceutical compositions comprising effective amounts of agonists of EEC, or other molecules capable of enhancing EEC activity, for use in treating conditions where it is desirable to enhance leukocyte trafficking, for example, in infection.

The invention further provides diagnostic assays and kits for the detection of EEC in cells and tissues comprising a purified EEC which may be used as a positive control, and anti-EEC antibodies. Such antibodies may be used in solution-based, membrane-based, or tissue-based technologies to detect any disease state or condition related to the expression of protein or expression of deletions or variants thereof.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 displays the nucleotide sequence for eosinophilic expressed chemokine, eec, and the predicted amino acid sequence of EEC.

Figure 2 shows the amino acid alignment of EEC with other human chemokines of the C-C family, including MIP-1a, SEQ ID NO: 3; MIP-1b, SEQ ID NO:4; MCP-1, SEQ ID NO:5; MCP-2, SEQ ID NO:6; MCP-3, SEQ ID NO:7; RANTES, SEQ ID NO:8; and Majority, SEQ ID NO:9. Alignments shown in Figures 2 and 5 were produced using the multisequence alignment program of DNASTAR software

"Nucleic acid sequence" as used herein refers to an oligonucleotide, nucleotide or polynucleotide sequence, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be double-stranded or single-stranded, whether representing the sense or antisense strand. As used herein "amino acid sequence" refers to peptide or protein sequences or portions thereof. As used herein, lower case "eec" refers to a nucleic acid sequence whereas upper case "EEC" refers to a protein sequence. As used herein, peptide nucleic acid (PNA) refers to a class of informational molecules that have a neutral "peptide like" backbone with nucleobases that allow molecules to hybridize to complementary DNA or RNA with higher affinity and specificity than corresponding oligonucleotides (PerSeptive Biosystems 1-800-899-5858).

As used herein, EEC encompasses EEC from any mammalian species, including bovine, ovine, murine, porcine, equine and preferably human sources, in naturally occurring or in variant form, or from any source, whether natural, synthetic, semi-synthetic or recombinant.

As used herein, "naturally occurring" refers to a EEC with an amino acid sequence found in nature, and "biologically active" refers to a EEC having structural, regulatory or biochemical functions of the naturally occurring EEC, including immunological activity. Naturally occurring EEC also encompasses those EECs arising from post-translational modifications of the polypeptide including but not limited to acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation. As used herein, "immunological activity" is defined as the capability of the natural, recombinant or synthetic EEC or any oligopeptide thereof, to induce a specific immune response in appropriate animals or cells and to bind with specific antibodies.

The term "derivative" as used herein refers to the chemical modification of an EEC. Illustrative of such modifications is replacement of hydrogen by an alkyl, acyl, or amino group. A EEC polypeptide derivative retains essential biological characteristics of a naturally occurring EEC. EEC derivative also refers to those EEC polypeptides derived from naturally occurring EEC by chemical modifications such as ubiquitination, labeling (e.g., with radionuclides, various enzymes, etc.), pegylation (derivatization with polyethylene glycol), or by insertion or substitution by chemical synthesis of amino acids such as ornithine, which do not normally occur in human proteins.

As used herein, the term "purified" refers to molecules, either nucleic

or amino acid sequences, that are removed from their natural environment and isolated or separated from at least one other component with which they are naturally associated.

"Recombinant variant EEC" refers to any EEC polypeptide differing from naturally occurring EEC by amino acid insertions, deletions, and substitutions, created using recombinant DNA techniques. Guidance in determining which amino acid residues may be replaced, added or deleted without abolishing activities of interest, such as cell adhesion and chemotaxis, may be found by comparing the sequence of the particular EEC with that of homologous cytokines and minimizing the number of amino acid sequence changes made in regions of high homology.

Preferably, amino acid "substitutions" are the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, such as the replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine, i.e., conservative amino acid replacements. "Insertions" or "deletions" are typically in the range of about 1 to 5 aa. The variation allowed may be experimentally determined by systematically making insertions, deletions, or substitutions of amino acids in an EEC molecule using recombinant DNA techniques and assaying the resulting recombinant variants for activity.

Where desired, a "signal or leader sequence" can direct the polypeptide through the membrane of a cell. Such a sequence may be naturally present on the polypeptides of the present invention or provided from heterologous protein sources by recombinant DNA techniques.

As used herein, an EEC "fragment," "portion," or "segment" refers is a stretch of amino acid residues which has sufficient length to display biologic and/or immunogenic activity and in preferred embodiments will contain at least about 5 amino acids, at least about 7 amino acids, at least about 8 to 13 amino acids, and, in additional embodiments, about 17 or more amino acids.

As used herein, an "oligonucleotide" or polynucleotide "fragment", "portion," or "segment" refers to any stretch of nucleic acids encoding EEC which is of sufficient length to use as a primer in polymerase chain reaction (PCR) or various hybridization procedures known to those of skill in the art, for the purpose of identifying or amplifying identical or related nucleic acids.

The present invention includes purified EEC polypeptides from natural or recombinant sources, vectors and host cells transformed with recombinant

nucleic acid molecules encoding EEC. Various methods for the isolation of the EEC polypeptides may be accomplished by procedures well known in the art. For example, such polypeptides may be purified by immunoaffinity chromatography by employing the antibodies provided by the present invention. Various other methods of protein purification well known in the art include those described in Deutscher M (1990) Methods in Enzymology, Vol 182, Academic Press, San Diego; and Scopes R (1982) Protein Purification: Principles and Practice. Springer-Verlag, NYC, both incorporated herein by reference.

As used herein the term "recombinant" refers to a polynucleotide which encodes EEC and is prepared using recombinant DNA techniques. The polynucleotide which encodes EEC may also include allelic or recombinant variants and mutants thereof.

As used herein the term "probe" or "nucleic acid probe" or "oligonucleotide probe" refers to a portion, fragment, or segment of eec that is capable of being hybridized to a desired target nucleotide sequence. A probe can be used to detect, amplify or quantify cDNAs or endogenous nucleic acid encoding EEC by employing conventional techniques in molecular biology. A probe may be of variable length, preferably from about 10 nucleotides up to several hundred nucleotides. As will be understood by those of skill in the art, hybridization conditions and probe design will vary depending upon the intended use. For example, a probe intended for use in PCR will be from about 15 to 60 nucleotides in length and may be part of a pool of degenerate probes, i.e., oligonucleotides which tolerate nucleotide mismatch but accommodate binding to an unknown sequence; whereas a probe for use in Southern or northern hybridizations may be a single, specific nucleotide sequence that is several hundred nucleotides in length. Nucleic acid probes may comprise portions of the sequence having fewer nucleotides than about 6 kb and usually fewer than about 1 kb. The oligonucletides and nucleic acid probes of the present invention may be used to determine whether nucleic acid encoding EEC is present in a cell or tissue or to isolate identical or similar nucleic acid sequences from chromosomal DNA as described by Walsh PS et al (1992 PCR Methods Appl 1:241-250).

Accordingly, a preferred probe for the specific detection of eec will comprise a polynucleotide or oligonucleotide fragment from a non-conserved nucleotide region of SEQ ID NO:1. As used herein the term "non-conserved nucleotide region" refers to a nucleotide region that is unique to SEQ ID NO:1 and does not comprise a region that is conserved in the family of C-C

chemokines. Probes may be single-stranded or double-stranded and may have specificity in solution, cell, tissue or membrane-based hybridizations including in situ and ELISA-like technologies. In an embodiment disclosed herein, a nucleotide probe for the detection of EEC encoding nucleotide sequences is derived from the nucleotide sequences encoding amino acid residues from amino acid residue position 22 to 63, inclusive, of SEQ ID NO:2.

Nucleic acid probes of the present invention may be derived from naturally occurring or recombinant single- or double-stranded nucleic acids or be chemically synthesized. They may be labeled by nick translation, Klenow fill-in reaction, PCR or other methods well known in the art. Probes of the present invention, their preparation and/or labeling are elaborated in Sambrook J et al (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, NY; or Ausubel FM et al (1989) Current Protocols in Molecular Biology, John Wiley & Sons, NYC, both incorporated herein by reference.

Alternatively, recombinant variant nucleotide sequences encoding the polypeptides of the present invention may be synthesized or identified through hybridization techniques known to those of skill in the art by making use of the "redundancy" in the genetic code. Various codon substitutions, such as the silent changes which produce various restriction sites, may be introduced to optimize cloning into a plasmid or viral vector or expression in a particular prokaryotic or eukaryotic system. Mutations may also be introduced to modify the properties of the polypeptide, to change ligand-binding affinities, interchain affinities, or polypeptide degradation or turnover rate.

Description

EEC Coding Sequences

The nucleotide sequence of human eec (SEQ ID NO:1) is shown in Figure 1. The coding region for EEC was initially identified within a cDNA library (Incyte cDNA library EOSIHET02) made from blood cells from a patient diagnosed with Hypereosinophilic Syndrome where it was found seven times in about 9576 usable sequences. A BLAST search (Basic Local Alignment Search Tool; Altschul SF (1993) J. Mol. Evol. 36: 290-300; Altschul SF et al (1990) J. Mol. Biol. 215:403-410) comparing the cDNAs of the EOSIHET02 library against the primate database of GenBank identified Incyte Clone 288236 as a non-exact match to a human 464.2 mRNA for a cytokine effector (GI G34750). Nucleotide sequences encoding EEC were also found one time in about 2553

sequences in a cDNA library (INCYTE UTRSNOT01) made from uterus tissue.

Because EEC is expressed in eosinophils, the nucleic acids (eec), polypeptides (EEC) and antibodies to EEC are useful in diagnostic assays based on chemokine production in cases of inflammation or disease affecting the number and function of eosinophils. Excessive expression of EEC can also activate monocytes, macrophages, basophils, T lymphocytes and/or other cells which respond to the chemokines and result in the production of abundant proteases and other molecules which can lead to tissue damage or destruction. Therefore, a diagnostic test for excess expression of EEC can accelerate diagnosis and proper treatment of eosinophilia, an abnormal condition caused by viral, bacterial, fungal or parasitic infections; mechanical injury associated with trauma; hereditary diseases such as allergies and asthma; infiltrative diseases such as leukemias and lymphomas; or other physiologic and pathologic problems associated with changes in the numbers of eosinophils.

Methods for DNA sequencing are well known in the art and employ such enzymes as the Klenow fragment of DNA polymerase I, Sequenase® (US Biochemical Corp, Cleveland OH)), Taq polymerase (Perkin Elmer,Norwalk CN), thermostable T7 polymerase (Amersham, Chicago IL), or combinations of recombinant polymerases and proofreading exonucleases such as the ELONGASE Amplification System marketed by Gibco BRL (Gaithersburg MD). Methods to extend the DNA from an oligonucleotide primer annealed to the DNA template of interest have been developed for both single-stranded and double-stranded templates. Chain termination reaction products were separated using electrophoresis and detected via their incorporated, labeled precursors. Recent improvements in mechanized reaction preparation, sequencing and analysis have permitted expansion in the number of sequences that can be determined per day. Preferably, the process is automated with machines such as the Hamilton Micro Lab 2200 (Hamilton, Reno NV), Peltier Thermal Cycler (PTC200; MJ Research, Watertown MA) and the ABI Catalyst 800 and 377 and 373 DNA sequencers (Perkin Elmer, Norwalk CN).

The quality of any particular cDNA library in which polynucleotides encoding EEC are found may be determined by performing a pilot scale analysis of the cDNAs and checking for percentages of clones containing vector, lambda or *E. coli* DNA, mitochondrial or repetitive DNA, and clones with exact or homologous matches to public databases.

The nucleotide sequences encoding EEC (or their complement) have numerous applications in techniques known to those skilled in the art of

molecular biology. These techniques include use as hybridization probes, use in the construction of oligomers for PCR, use for chromosome and gene mapping, use in the recombinant production of EEC, and use in generation of anti-sense DNA or RNA, their chemical analogs and the like. Uses of nucleotides encoding EEC disclosed herein are exemplary of known techniques and are not intended to limit their use in any technique known to a person of ordinary skill in the art. Furthermore, the nucleotide sequences disclosed herein may be used in molecular biology techniques that have not yet been developed, provided the new techniques rely on properties of nucleotide sequences that are currently known, e.g., the triplet genetic code, specific base pair interactions, etc.

It will be appreciated by those skilled in the art that as a result of the degeneracy of the genetic code, a multitude of EEC-encoding nucleotide sequences, some bearing minimal homology to the nucleotide sequence of any known and naturally occurring gene may be produced. The invention has specifically contemplated each and every possible variation of nucleotide sequence that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the nucleotide sequence of naturally occurring eec, and all such variations are to be considered as being specifically disclosed.

Although the nucleotide sequences which encode EEC and/or its variants are preferably capable of hybridizing to the nucleotide sequence of the naturally occurring eec under stringent conditions, it may be advantageous to produce nucleotide sequences encoding EEC or its derivatives possessing a substantially different codon usage. Codons can be selected to increase the rate at which expression of the peptide occurs in a particular prokaryotic or eukaryotic expression host in accordance with the frequency with which particular codons are utilized by the host. Other reasons for substantially altering the nucleotide sequence encoding EEC and/or its derivatives without altering the encoded amino acid sequence include the production of RNA transcripts having more desirable properties, such as a greater half-life, than transcripts produced from the naturally occurring sequence.

Nucleotide sequences encoding EEC may be joined to a variety of other nucleotide sequences by means of well established recombinant DNA techniques (cf Sambrook J et al. supra). Useful nucleotide sequences for joining to eec include an assortment of cloning vectors, e.g., plasmids, cosmids, lambda phage derivatives, phagemids, and the like, that are well known in the art.

Vectors of interest include expression vectors, replication vectors, probe generation vectors, sequencing vectors, and the like. In general, vectors of interest may contain an origin of replication functional in at least one organism, convenient restriction endonuclease sensitive sites, and selectable markers for the host cell.

One aspect of the subject invention is to provide for eec-specific nucleic acid hybridization probes capable of hybridizing with naturally occurring nucleotide sequences encoding EEC. Such probes may also be used for the detection of similar chemokine encoding sequences and should preferably contain at least 50% of the nucleotides from a C-C encoding sequence. The hybridization probes of the subject invention may be derived from the nucleotide sequences of the SEQ ID NO:1 or from genomic sequences including promoters, enhancer elements and/or possible introns of the respective naturally occurring eecs. Hybridization probes may be labeled by a variety of reporter groups, including radionuclides such as 32P or 35S, or enzymatic labels such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems, and the like.

PCR as described US Patent Nos 4,683,195; 4,800,195; and 4,965,188 provides additional uses for oligonucleotides based upon the nucleotide sequence which encodes EEC. Such probes used in PCR may be of recombinant origin, may be chemically synthesized, or a mixture of both and comprise a discrete nucleotide sequence for diagnostic use or a degenerate pool of possible sequences for identification of closely related genomic sequences.

Other means of producing specific hybridization probes for eec DNAs include the cloning of nucleic acid sequences encoding EEC or EEC derivatives into vectors for the production of mRNA probes. Such vectors are known in the art and are commercially available and may be used to synthesize RNA probes in vitro by means of the addition of the appropriate RNA polymerase as T7 or SP6 RNA polymerase and the appropriate radioactively labeled nucleotides.

It is now possible to produce a DNA sequence, or portions thereof, encoding EEC and their derivatives entirely by synthetic chemistry, after which the gene can be inserted into any of the many available DNA vectors using reagents, vectors and cells that are known in the art at the time of the filing of this application. Moreover, synthetic chemistry may be used to introduce mutations into the eec sequences or any portion thereof.

The nucleotide sequence can be used in an assay to detect inflammation or disease associated with abnormal levels of expression of EEC. The

nucleotide sequence can be labeled by methods known in the art and added to a fluid or tissue sample from a patient under hybridizing conditions. After an incubation period, the sample is washed with a compatible fluid which optionally contains a dye (or other label requiring a developer) if the nucleotide has been labeled with an enzyme. After the compatible fluid is rinsed off, the dye is quantitated and compared with a standard. If the amount of dye is significantly elevated, the nucleotide sequence has hybridized with the sample, and the assay indicates the presence of inflammation and/or disease.

The nucleotide sequence for eec can be used to construct hybridization probes for mapping that gene. The nucleotide sequence provided herein may be mapped to a particular chromosome or to specific regions of that chromosome using well known genetic and/or chromosomal mapping techniques. These techniques include in situ hybridization, linkage analysis against known chromosomal markers, hybridization screening with libraries, flow-sorted chromosomal preparations, or artificial chromosome constructions YAC or P1 constructions. The technique of fluorescent in situ hybridization of chromosome spreads has been described, among other places, in Verma et al (1988) Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York City.

Fluorescent in situ hybridization of chromosomal preparations and other physical chromosome mapping techniques may be correlated with additional genetic map data. Examples of genetic map data can be found in the 1994 Genome Issue of Science (265:1981f). Correlation between the location of eec on a physical chromosomal map and a specific disease (or predisposition to a specific disease) can help delimit the region of DNA associated with that genetic disease. The nucleotide sequence of the subject invention may be used to detect differences in gene sequence between normal and carrier or affected individuals.

Expression of EEC

Nucleotide sequences encoding EEC may be used to produce purified EEC using well known methods of recombinant DNA technology. Among the many publications that teach methods for the expression of genes after they have been isolated is Goeddel (1990) Gene Expression Technology, Methods and Enzymology, Vol 185, Academic Press, San Diego CA. EEC may be expressed in a variety of host cells, either prokaryotic or eukaryotic. Host cells may be from the same species in which eec nucleotide sequences are endogenous or from a different species. Advantages of producing EEC by recombinant DNA

technology include obtaining adequate amounts of the protein for purification and the availability of simplified purification procedures. Expression of eec may be accomplished by subcloning the cDNAs into appropriate expression vectors and transfecting the vectors into appropriate expression hosts. As described in Example VII, a preferred expression vector is one which provides for expression of a fusion protein comprising EEC and contains nucleic acid encoding 6 histidine residues followed by thioredoxin and an enterokinase cleavage site. The histidine residues facilitate purification on IMIAC (immobilized metal ion affinity chromatography as described in Porath et al. (1992) Protein Expression and Purification 3:263-281) while the enterokinase cleavage site provides a means for purifying the chemokine from the fusion protein. The cloning vector previously used for the generation of the tissue library also provide for expression of the eec sequence in E. coli.

Since cDNA clone inserts are generated by an essentially random process, there is one chance in three that the included cDNA will lie in the correct frame for proper translation. If the cDNA is not in the proper reading frame, it can be obtained by deletion or insertion of the appropriate number of bases by well known methods including in vitro mutagenesis, digestion with exonuclease III or mung bean nuclease, or oligonucleotide linker inclusion.

The eec cDNA can be shuttled into other vectors known to be useful for expression of protein in specific hosts. Oligonucleotide amplimers containing cloning sites as well as a segment of DNA sufficient to hybridize to stretches at both ends of the target cDNA (25 bases) can be synthesized chemically by standard methods. These primers can then used to amplify the desired gene segments by PCR. The resulting new gene segments can be digested with appropriate restriction enzymes under standard conditions and isolated by gel electrophoresis. Alternately, similar gene segments can be produced by digestion of the cDNA with appropriate restriction enzymes and filling in the missing gene segments with chemically synthesized oligonucleotides. Segments of the coding sequence from more than one gene can be ligated together and cloned in appropriate vectors to optimize expression of recombinant sequence.

Suitable expression hosts for such chimeric molecules include but are not limited to mammalian cells such as Chinese Hamster Ovary (CHO) and human 293 cells, insect cells such as Sf9 cells, yeast cells such as Saccharomyces cerevisiae, and bacteria such as E. coli. For each of these cell systems, a useful expression vector may also include an origin of replication to allow

propagation in bacteria and a selectable marker such as the ß-lactamase antibiotic resistance gene to allow selection in bacteria. In addition, the vectors may include a second selectable marker such as the neomycin phosphotransferase gene to allow selection in transfected eukaryotic host cells. Vectors for use in eukaryotic expression hosts may require RNA processing elements such as 3' polyadenylation sequences if such are not part of the cDNA of interest.

Additionally, the vector may contain promoters or enhancers which increase gene expression. Such promoters are host specific and include MMTV, SV40, or metallothionine promoters for CHO cells; trp, lac, tac or T7 promoters for bacterial hosts, or alpha factor, alcohol oxidase or PGH promoters for yeast. Transcription enhancers, such as the rous sarcoma virus (RSV) enhancer, may be used in mammalian host cells. Once homogeneous cultures of recombinant cells are obtained through standard culture methods, large quantities of recombinantly produced EEC can be recovered from the conditioned medium and analyzed using chromatographic methods known in the art.

Cells transformed with DNA encoding EEC may be cultured under conditions suitable for the expression of chemokines and recovery of the protein from the cell culture. EEC produced by a recombinant cell may be secreted or may be contained intracellularly, depending on the particular genetic construction used. In general, it is more convenient to prepare recombinant proteins in secreted form. Purification steps vary with the production process and the particular protein produced.

EEC may be expressed as a chimeric protein with one or more additional polypeptide domains added to facilitate protein purification. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle WA). The inclusion of a cleavable linker sequence such as Factor XA or enterokinase(Invitrogen, San Diego CA) between the purification domain and the eec sequence may be useful to facilitate expression of EEC.

In addition to recombinant production, fragments of EEC may be produced by direct peptide synthesis using solid-phase techniques (cf Stewart et al (1969) Solid-Phase Peptide Synthesis, WH Freeman Co, San Francisco CA; Merrifield J (1963) J Am Chem Soc 85:2149-2154. In vitro protein synthesis

may be performed using manual techniques or by automation. Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Foster City, California CA) in accordance with the instructions provided by the manufacturer. Various fragments of EEC may be chemically synthesized separately and combined using chemical methods to produce the full length molecule.

EEC Antibodies

EEC for antibody induction does not require biological activity; however, the protein must be immunogenic. Peptides used to induce specific antibodies may have an amino acid sequence consisting of at least five amino acids, preferably at least 10 amino acids. They should mimic a portion of the amino acid sequence of the protein and may contain the entire amino acid sequence of a small naturally occurring molecule such as EEC. Short stretches of EEC amino acids may be fused with those of another protein such as keyhole limpet hemocyanin and the chimeric molecule used for antibody production.

Antibodies specific for EEC may be produced by inoculation of an appropriate animal with the polypeptide or an antigenic fragment. An antibody is specific for EEC if it is produced against an epitope of the polypeptide and binds to at least part of the natural or recombinant protein. Antibody production includes not only the stimulation of an immune response by injection into animals, but also analogous steps in the production of synthetic antibodies or other specific-binding molecules such as the screening of recombinant immunoglobulin libraries (cf Orlandi R et al (1989) PNAS 86:3833-3837, or Huse WD et al (1989) Science 256:1275-1281) or the in vitro stimulation of lymphocyte populations. Current technology (Winter G and Milstein C (1991) Nature 349:293-299) provides for a number of highly specific binding reagents based on the principles of antibody formation. These techniques may be adapted to produce molecules specifically binding EECs.

Various methods are known to those of skill in the art for preparing monoclonal and polyclonal antibodies to EEC. In one approach, denatured EEC from the reverse phase HPLC separation is obtained and used to immunize mice or rabbits using techniques known to those of skill in the art. About 100 micrograms are adequate for immunization of a mouse, while up to 1 mg might be used for immunization of a rabbit. For identifying mouse hybridomas, the denatured protein can be radioiodinated and used to screen potential murine B-cell hybridomas for those which produce antibody. This procedure requires

only small quantities of protein, such that 20 mg would be sufficient for labeling and screening of several thousand clones.

In another approach, the amino acid sequence of EEC, as deduced from translation of the cDNA sequence, is analyzed to determine regions of high immunogenicity. Oligopeptides comprising hydrophilic regions, as shown in Figure 3, are synthesized and used in suitable immunization protocols to raise antibodies. Analysis to select appropriate epitopes is described by Ausubel FM et al (1989, Current Protocols in Molecular Biology, John Wiley & Sons, NYC). The optimal amino acid sequences for immunization are usually at the C-terminus, the N-terminus and those intervening, hydrophilic regions of the polypeptide which are likely to be exposed to the external environment when the protein is in its natural conformation.

Typically, selected peptides, about 15 residues in length, are synthesized using an Applied Biosystems Peptide Synthesizer Model 431A using fmoc-chemistry and coupled to keyhole limpet hemocyanin (KLH, Sigma) by reaction with M-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; cf. Ausubel FM et al, supra). If necessary, a cysteine may be introduced at the N-terminus of the peptide to permit coupling to KLH and animals can be immunized with the peptide-KLH complex in complete Freund's adjuvant. The resulting antisera can be tested for antipeptide activity by binding the peptide to plastic, blocking with 1% BSA, reacting with antisera, washing and reacting with labeled (radioactive or fluorescent), affinity purified, specific goat anti-rabbit IgG.

Hybridomas may also be prepared and screened using standard techniques. Hybridomas of interest can be detected by screening with labeled EEC to identify those fusions producing the monoclonal antibody with the desired specificity. In a typical protocol, wells of plates (FAST; Becton-Dickinson, Palo Alto, CA) are coated with affinity purified, specific rabbit-anti-mouse (or suitable anti-species Ig) antibodies at 10 mg/ml. The coated wells are blocked with 1% BSA, washed and exposed to supernatants from hybridomas. After incubation, the wells are exposed to labeled EEC at 1 mg/ml. Clones producing antibodies will bind a quantity of labeled EEC which is detectable above background. Such clones can be expanded and subjected to 2 cycles of cloning at limiting dilution (1 cell/3 wells). Cloned hybridomas are injected into pristane-treated mice to produce ascites, and monoclonal antibody can be purified from mouse ascitic fluid by affinity chromatography using Protein A. Monoclonal antibodies with affinities of at least 10^8 M^{-1}, preferably 10^9 to 10^{10} or stronger, will typically be made by standard

procedures as described in Harlow and Lane (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory New York; and in Goding (1986) Monoclonal Antibodies: Principles and Practice, Academic Press, New York City, both incorporated herein by reference.

Uses of Nucleotides and Amino Acid Sequences for EEC

An additional embodiment of the subject invention is the use of EEC specific antibodies, inhibitors, receptors or their analogs as bioactive agents to treat eosinophilia, inflammation or disease involving an altered number of eosinophils including, but not limited to viral, bacterial, fungal or parasitic infections; mechanical injury associated with trauma; hereditary diseases such as allergies and asthma; infiltrative diseases such as leukemias and lymphomas; or other physiologic and pathologic problems associated with changes in the numbers of eosinophils.

Knowledge of the correct, complete cDNA sequence of the novel expressed chemokine gene will enable its use in antisense technology in the investigation of gene function. Oligonucleotides, genomic or cDNA fragments comprising the antisense strand of eec can be used either in vitro or in vivo to inhibit expression of the protein. Such technology is now well known in the art, and probes can be designed at various locations along the nucleotide sequence. By treatment of cells or whole test animals with such antisense sequences, the gene of interest can effectively be turned off. Frequently, the function of the gene can be ascertained by observing behavior at the cellular, tissue or organismal level (e.g. lethality, loss of differentiated function, changes in morphology, etc.).

In addition to using sequences constructed to interrupt transcription of the open reading frame, modifications of gene expression can be obtained by designing antisense sequences to intron regions, promoter/enhancer elements, or even to trans-acting regulatory genes. Similarly, inhibition can be achieved using Hogeboom base-pairing methodology, also known as "triple helix" base pairing.

Antibodies, inhibitors, receptors or antagonists of EEC (or other treatments for excessive chemokine production, hereinafter abbreviated TEC), can provide different effects when administered therapeutically. TECs will be formulated in a nontoxic, inert, pharmaceutically acceptable aqueous carrier medium preferably at a pH of about 5 to 8, more preferably 6 to 8, although the pH may vary according to the characteristics of the antibody, inhibitor, receptor or antagonist being formulated and the condition to be treated. Characteristics of TEC include solubility of the molecule,

half-life and antigenicity/immuno-genicity; these and other characteristics may aid in defining an effective carrier. Native human proteins are preferred as TECs, but organic or synthetic molecules resulting from drug screens may be equally effective in particular situations.

TECs may be delivered by known routes of administration including but not limited to topical creams and gels; transmucosal spray and aerosol, transdermal patch and bandage; injectable, intravenous and lavage formulations; and orally administered liquids and pills, particularly formulated to resist stomach acid and enzymes. The particular formulation, exact dosage, and route of administration will be determined by the attending physician and will vary according to each specific situation.

Such determinations are made by considering multiple variables such as the condition to be treated, the TEC to be administered, and the pharmacokinetic profile of the particular TEC. Additional factors which may be taken into account include disease state (e.g. severity) of the patient, age, weight, gender, diet, time of administration, drug combination, reaction sensitivities, and tolerance/response to therapy. Long acting TEC formulations might be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular TEC.

Normal dosage amounts may vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature; see US Patent No. 4,657,760; 5,206,344; or 5,225,212. It is anticipated that different formulations will be effective for different TECs and that administration targeting the eosinophil may necessitate delivery in a manner different from that to another organ or tissue.

It is contemplated that conditions or diseases of the eosinophil which activate monocytes, macrophages, basophils, eosinophils or other leukocytes may precipitate damage that is treatable with TECs. Eosinophilia may be specifically diagnosed by the tests discussed above, and such testing should be performed in suspected cases of viral, bacterial, fungal or parasitic infections as recited herein; mechanical injuries associated with trauma; hereditary diseases such as allergies, asthma, and rheumatoid arthritis; cancers such as the recited carcinomas, leukemias, and lymphomas; or other physiologic or pathologic problems associated with changes in the numbers of eosinophils.

All publications and patents mentioned in the above specification are

herein incorporated by reference. The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Indeed, various modifications of the above described modes for carrying out the invention which are readily apparent to those skilled in the field of molecular biology or related fields are intended to be within the scope of the following claims.

The examples below are provided to illustrate the subject invention. These examples are provided by way of illustration and are not included for the purpose of limiting the invention.

INDUSTRIAL APPLICABILITY

I Isolation of mRNA and Construction of cDNA Libraries

The eec sequence was identified among the sequences of a human eosinophil library. The eosinophils used for this library were obtained via aphoresis of a 56 year old Caucasian male patient at Mayo Clinic (Rochester MN) who had been diagnosed with Hypereosinophilic Syndrome. The cells were washed twice in phosphate buffered saline and lysed immediately in a buffer containing guanidinium isothiocyanate. The lysate was centrifuged over a CsCl cushion, ethanol precipitated, resuspended in water and DNase treated for 15 min at 37°C. The RNA was extracted with phenol chloroform and precipitated with ethanol. Polyadenylated messages were isolated using Qiagen Oligotex (QIAGEN Inc, Chatsworth CA), and the cDNA library was constructed by Stratagene (11011 North Torrey Pines Road, La Jolla CA 92037).

First strand cDNA synthesis was accomplished using an oligo d(T) primer/linker which also contained an XhoI restriction site. Second strand synthesis was performed using a combination of DNA polymerase I, E. coli ligase and RNase H, followed by the addition of an EcoRI adaptor to the blunt ended cDNA. The EcoRI adapted, double-stranded cDNA was then digested with XhoI restriction enzyme, extracted with phenol chloroform, and fractionated by size on Sephacryl S400. DNA of the appropriate size was then ligated to dephosphorylated Lambda Zap® arms (Stratagene) and packaged using Gigapack extracts (Stratagene). pBluescript (Stratagene) phagemid DNAs were excised en masse from the eosinophil library and individual plasmid DNAs were made using Miniprep Kits supplied by Advanced Genetic Technologies Corporation (Gaithersburg MD).

These kits provide a 96-well format and enough reagents for 960 purifications. The recommended protocol supplied with each kit has been employed except for the following changes. First, the 96 wells are each filled with only 1 ml of sterile terrific broth with carbenicillin at 25 mg/L

and glycerol at 0.4%. After the wells are inoculated, the bacteria are cultured for 24 hours and lysed with 60 µl of lysis buffer. A centrifugation step (2900 rpm for 5 minutes) is performed before the contents of the block are added to the primary filter plate. The optional step of adding isopropanol to TRIS buffer is not routinely performed. After the last step in the protocol, samples are transferred to a Beckman 96-well block for storage.

The quality of the cDNA library was determined by performing a pilot scale analysis of 192 cDNAs and checking for percentages of clones containing vector alone, mitochondrial or repetitive DNA sequences and clones originating from lambda or E. coli DNA. The numbers of exact/homologous matches to public databases, as well as the number of unique sequences, i.e., those having no known match in any available database, were also recorded.

II Isolation of cDNA Clones

The phagemid forms of individual cDNA clones were obtained by the in vivo excision process, in which XL1-BLUE was coinfected with an f1 helper phage. Proteins derived from both lambda phage and f1 helper phage initiated new DNA synthesis from defined sequences on the lambda target DNA and create a smaller, single-stranded circular phagemid DNA molecule that includes all DNA sequences of the pBluescript plasmid and the cDNA insert. The phagemid DNA was released from the cells and purified, then used to re-infect fresh bacterial host cells (SOLR, Stratagene Inc), where the double-stranded phagemid DNA was produced. Because the phagemid carries the gene for ß-lactamase, the newly transformed bacteria were selected on medium containing ampicillin.

Phagemid DNA was purified using the QIAWELL-8 Plasmid Purification System from QIAGEN® DNA Purification System. This technique provides a rapid and reliable high-throughput method for lysing the bacterial cells and isolating highly purified phagemid DNA. The DNA eluted from the purification resin was suitable for DNA sequencing and other analytical manipulations.

The cDNA inserts from random isolates of the human eosinophilic library were sequenced in part. The cDNAs were sequenced by the method of Sanger F. and AR Coulson (1975; J. Mol. Biol.94:441f), using a Hamilton Micro Lab 2200 (Hamilton, Reno NV) in combination with four Peltier Thermal Cyclers (PTC200 from MJ Research, Watertown MA) and Applied Biosystems 377 or 373 DNA Sequencing Systems (Perkin Elmer) and reading frame determined.

III Homology Searching of cDNA Clones and Deduced Proteins

Each sequence so obtained was compared to sequences in GenBank using a search algorithm developed by Applied Biosystems Inc. and incorporated into

the INHERIT™ 670 Sequence Analysis System. In this algorithm, Pattern Specification Language (developed by TRW Inc.) was used to determine regions of homology. The three parameters that determine how the sequence comparisons run were window size, window offset, and error tolerance. Using a combination of these three parameters, the DNA database was searched for sequences containing regions of homology to the query sequence, and the appropriate sequences were scored with an initial value. Subsequently, these homologous regions were examined using dot matrix homology plots to distinguish regions of homology from chance matches. Smith-Waterman alignments were used to display the results of the homology search.

Peptide and protein sequence homologies were ascertained using the INHERIT 670 Sequence Analysis System in a way similar to that used in DNA sequence homologies. Pattern Specification Language and parameter windows were used to search protein databases for sequences containing regions of homology which were scored with an initial value. Dot-matrix homology plots were examined to distinguish regions of significant homology from chance matches.

BLAST, which stands for Basic Local Alignment Search Tool (Altschul SF (1993) J Mol Evol 36:290-300; Altschul, SF et al (1990) J Mol Biol 215:403-10), was used to search for local sequence alignments . BLAST produces alignments of both nucleotide and amino acid sequences to determine sequence similarity. Because of the local nature of the alignments, BLAST is especially useful in determining exact matches or in identifying homologs. BLAST is useful for matches which do not contain gaps. The fundamental unit of BLAST algorithm output is the High-scoring Segment Pair (HSP).

An HSP consists of two sequence fragments of arbitrary but equal lengths whose alignment is locally maximal and for which the alignment score meets or exceeds a threshold or cutoff score set by the user. The BLAST approach is to look for HSPs between a query sequence and a database sequence, to evaluate the statistical significance of any matches found, and to report only those matches which satisfy the user-selected threshold of significance. The parameter E establishes the statistically significant threshold for reporting database sequence matches. E is interpreted as the upper bound of the expected frequency of chance occurrence of an HSP (or set of HSPs) within the context of the entire database search. Any database sequence whose match satisfies E is reported in the program output.

The nucleotide and amino acid sequences for the entire coding region of the the eosinophil expressed chemokine, EEC, are shown in Figure 1.

IV Identification and Full Length Sequencing of the Genes

From all of the randomly picked and sequenced clones of the human eosinophil library, the eec sequence was homologous to but clearly different from any known C-C chemokine molecule. The complete nucleotide sequence for eec was translated, and the in-frame translation is shown in Figure 1. When all three possible predicted translations of the sequence were searched against protein databases such as SwissProt and PIR, no exact matches were found to the possible translations of eec. Figure 2 shows the comparison of the EEC amino acid sequence with those of other C-C chemokine molecules. The substantial regions of homology among these molecules which includes the definitive C-C motif are shaded. Hydrophobicity plots for EEC are shown as Figure 3. The phylogenetic analysis (Figure 4) shows how closely eec is related to other well characterized human C-C chemokines. The most related of these molecules cluster together at the right hand side of the figure.

V Antisense analysis

The EEC sequence, or any part thereof, is used to inhibit in vivo or in vitro expression of endogenous EEC. Although use of antisense oligonucleotides, consisting of about 20 base-pairs, is specifically described, essentially the same procedure is used with larger cDNA fragments. An oligonucleotide based on the coding sequence of EEC is used to inhibit expression of endogenous EEC. Using Oligo 4.0, the complementary oligonucleotide is designed from the conserved 5' sequence and used to inhibit either transcription, by preventing promoter binding to the upstream nontranslated sequence, or translation of an EEC transcript by preventing the ribosome from binding to the mRNA.

VI Expression of EEC

The nucleotide sequences encoding EEC were cloned into an expression vector that comprises a T7 promoter followed by an initiating methionine codon (ATG), followed by six histidine codons, followed by the TrxA gene of E.coli (which encodes thioredoxin), followed by a sequence coding for an enterokinase cleavage site and nucleotide sequences encoding EEC. Empirical studies associated with cleavage of signal sequences indicate that cleavage occurs at or near the C-terminal end of a predicted hydrophobic region located at the N-terminus of the full length protein. The hydrophobicity profile of EEC is shown in Figure 3 and based on this profile, residue 21 of SEQ ID NO:2 (Alanine), appears to be the N-terminal amino acid residue for expression of mature EEC. The presence of a N-terminal residue 50 amino acid residues from the characteristic C-C residues of a C-C chemokine may reflect

an N-terminal extension to EEC that imparts novel activity.

The expression vectors described above containing the 6 histidine codons were used to transform a host cell, the host cell culture was induced with IPTG and the expressed protein was subjected to denaturing SDS poly acrylamide gel electrophoresis. Nucleic acid from the expression vector was partially purified using the miniprep procedure of Sambrook supra which produced super-coiled DNA. About 100 ng of DNA were used to transform the host bacterial cell, W3110/DE3. W3110/DE3 was constructed using W3110 from the ATCC and the lambda DE3 lysogenization kit commercially available from Novagen. DE3 lysogens are often less competent than their parent, W3110, and are adapted to use super-coiled DNA for efficient transformation. A single transformant from each chemokine transformation was selected and used to inoculate a 5 ml culture of L-broth containing ampicillin. Each 5 ml culture was grown overnight (12-15 hours) at 37 degrees C. with shaking. The next day, 1 ml of the overnight culture was used to inoculate a 100 ml culture of L-broth with ampicillin in a 500 ml flask and allowed to grow at 37 degrees C. with shaking until the OD600 of the culture reached 0.4-0.6. If inoculated cells are allowed to grow past an OD600 of 0.6, they will begin to reach stationary phase and induction levels will be reduced.

At the time of inoculation, a 5 ml sample was removed, placed on ice and used as a pre-induction (or 0 hour) sample. When the cell culture reached an OD600 of 0.6, 400µl of an 100mM IPTG stock solution was added for a final concentration of 0.4mM. The cultures were allowed to grow for 3 hours at 37 degrees C. with shaking. Analysis of induction was determined by sampling 5 ml aliquots of the culture at 1 hour intervals up to 6 hours and analysing on a denaturing SDS poly acrylamide gel electrophoresis. The fusion protein appeared to accumulate in the insoluble fraction of the cells

Maximal induction of EEC occurred by 2 hours. Growth beyond 4 hours resulted in lysis in the culture and overall reduced yields of the desired protein due to proteolysis. Five ml aliquots of the cell cultures were obtained at 0, 1 and 2 hours and centrifuged for 5 minutes at 3000 RPM at 4 degrees C. The supernatant was aspirated and the pellets were subjected to a freeze-thaw step to help lyse the cells. The pellet was resuspended in TE [10mM Tris-HCL pH 8.0, 1mM EDTA pH 8.0] at 4 degrees C. at a volume calculated as: vol TE(ul) = (OD600)(250), and an equivalent volume of 2X SDS Sample Loading Buffer (Novex) was added to each sample. The samples were boiled for 5 minutes and 10µl of each sample was loaded per lane.

The expected molecular weight of the fusion protein comprising H6-Trx-

EEC is 19,233 Daltons. Analysis of the expressed EEC on a 14% SDS-polyacrylamide gel shows an apparent molecular weight of about 20kDa. A second fusion protein lacking the six histidine residues was constructed and expressed and has an expected molecular weight of 18,410 Daltons. The EEC protein appears predominantly in the insoluble fraction of the cell lysates.

VII Isolation of Recombinant EEC

EEC is expressed as a chimeric protein having six histidines followed by thioredoxin (TrxA of E.coli) with an enterokinase cleavage site between the TrxA protein and EEC. The histidines are added to facilitate protein purification. The presence of the histidines allows for purification on IMIAC chromatography (Porath supra).

VIII Diagnostic Test Using EEC Specific Antibodies

Particular EEC antibodies are useful for the diagnosis of prepathologic conditions, and chronic or acute diseases which are characterized by differences in the amount or distribution of EEC. EEC was initially found in the human eosinophil library and is diagnostic for abnormalities or pathologies associated with leukocyte trafficking and eosinophils.

Diagnostic tests for EEC use the antibody and a label to detect EEC in human body fluids, tissues or extracts of such tissues. The polypeptides and antibodies of the present invention are used with or without modification. The polypeptides and antibodies are labeled by joining them, either covalently or noncovalently, with a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and have been reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent agents, chemiluminescent agents, magnetic particles and the like. Patents teaching the use of such labels include US Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced as shown in US Patent No. 4,816,567, incorporated herein by reference.

A variety of protocols for measuring soluble or membrane-bound EEC, using either polyclonal or monoclonal antibodies specific for the respective protein are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). A two-site monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on EEC is preferred, but a competitive binding assay may be employed. These assays are described, among other places, in Maddox, DE et al (1983, J Exp Med 158:1211).

IX Purification of Native EEC Using Specific Antibodies

Native or recombinant EEC is purified by immunoaffinity chromatography using antibodies specific for EEC. An immunoaffinity column is constructed by covalently coupling the anti-EEC antibody to an activated chromatographic resin.

Polyclonal immunoglobulins are prepared from immune sera either by precipitation with ammonium sulfate or by purification on immobilized Protein A (Pharmacia LKB Biotechnology, Piscataway, NJ). Monoclonal antibodies are prepared from mouse ascites fluid by ammonium sulfate precipitation or chromatography on immobilized Protein A. Partially purified immunoglobulin is covalently attached to a chromatographic resin such as CnBr-activated Sepharose (Pharmacia LKB Biotechnology). The antibody is coupled to the resin, the resin is blocked, and the derivative resin is washed according to the manufacturer's instructions.

Such immunoaffinity columns are utilized in the purification of EEC by preparing a fraction from cells containing EEC in a soluble form. This preparation is derived by solubilization of the whole cell or of a subcellular fraction obtained via differential centrifugation by the addition of detergent or by other methods well known in the art. Alternatively, soluble EEC containing a signal sequence may be secreted in useful quantity into the medium in which the cells are grown.

A soluble EEC-containing preparation is passed over the immunoaffinity column, and the column is washed under conditions that allow the preferential absorbance of chemokines (eg, high ionic strength buffers in the presence of detergent). The column is eluted under conditions that disrupt antibody/chemokine binding (e.g., a buffer of pH 2-3 or a high concentration of a chaotrope such as urea or thiocyanate ion), and EEC is collected.

X EEC Induced Chemotaxis or Cell Activation

The chemotactic activity of EEC is measured in 48-well microchemotaxis chambers (Falk WR et al (1980) J Immunol Methods 33:239). In each well, two compartments are separated by a filter that allows the passage of cells in response to a chemical gradient. Cell culture medium such as RPMI 1640 (Sigma, St. Louis MO) containing the expressed chemokine is placed on one side of a filter, usually polycarbonate, and cells suspended in the same media are placed on the opposite side of the filter. Sufficient incubation time is allowed for the cells to traverse the filter in response to the concentration gradient across the filter. Filters are recovered from each well, and cells adhering to the side of the filter facing the chemokine are

typed and quantified.

The specificity of the chemoattraction is determined by performing the chemotaxis assay on specific populations of cells. First, blood cells obtained from venipuncture are fractionated by density gradient centrifugation and the chemotactic activity of EEC is tested on enriched populations of neutrophils, peripheral blood mononuclear cells, monocytes and lymphocytes. Optionally, such enriched cell populations are further fractionated using CD8+ and CD4+ specific antibodies for negative selection of CD4+ and CD8+ enriched T-cell populations, respectively.

Another assay elucidates the chemotactic effect of EEC on activated T-cells. Unfractionated T-cells or fractionated T-cell subsets are cultured for 6 to 8 hours in tissue culture vessels coated with CD-3 antibody. After this CD-3 activation, the chemotactic activity of EEC is tested as described above. Many other methods for obtaining enriched cell populations are known in the art.

Some chemokines also produce a non-chemotactic cell activation of neutrophils and monocytes. This is tested via standard measures of neutrophil activation such as actin polymerization, increase in respiratory burst activity, degranulation of the azurophilic granule and mobilization of Ca^{++} as part of the signal transduction pathway. The assay for mobilization of Ca^{++} involves preloading neutrophils with a fluorescent probe whose emission characteristics have been altered by Ca^{++} binding. When the cells are exposed to an activating stimulus, Ca^{++} flux is determined by observation of the cells in a fluorometer. The measurement of Ca^{++} mobilization has been described in Grynkievicz G et al (1985) J Biol Chem 260:3440, and McColl S et al (1993) J Immunol 150:4550-4555, incorporated herein by reference.

Degranulation and respiratory burst responses are also measured in monocytes (Zachariae COC et al. (1990) J Exp Med 171: 2177-82). Further measures of monocyte activation are regulation of adhesion molecule expression and cytokine production (Jiang Y et al (1992) J Immunol 148: 2423-8). Expression of adhesion molecules also varies with lymphocyte activation (Taub D et al (1993) Science 260: 355-358).

XI Drug Screening

EEC, or biologically active fragments thereof, are used for screening compounds in any of a variety of drug screening techniques. The chemokine polypeptide or fragment employed in such a test may either be free in solution, affixed to a solid support, borne on a cell surface or located intracellularly. One method of drug screening utilizes eukaryotic or

prokaryotic host cells which are stably transformed with recombinant nucleic acids expressing the polypeptide or fragment. Drugs are screened against such transformed cells in competitive binding assays. Such cells, either in viable or fixed form, can be used for standard binding assays. One may measure, for example, the formation of complexes between EEC and the agent being tested. Alternatively, one can examine the diminution in complex formation between EEC and its target cell, monocyte, etc. caused by the agent being tested.

Thus, the present invention provides methods of screening for drugs or any other agents which can affect inflammation and disease. These methods comprise contacting such an agent with a EEC polypeptide or fragment thereof and assaying (i) for the presence of a complex between the agent and the EEC polypeptide or fragment, or (ii) for the presence of a complex between the EEC polypeptide or fragment and the cell, by methods well known in the art. Typically in such competitive binding assays, the chemokine polypeptide or fragment is labeled. After suitable incubation, free EEC polypeptide or fragment is separated from that present in bound form, and the amount of free or uncomplexed label is a measure of the ability of the particular agent to bind to EEC or to interfere with the EEC and agent complex. Another embodiment of the present invention relates to a method of screening a plurality of compounds for specific binding affinity with the polypeptide of Claim 8 or any portion thereof, comprising the steps of: a) providing a plurality of compounds; b) combining Eosinophil Spleen Expressed Chemokine (EEC) with each of a plurality of compounds for a time sufficient to allow binding under suitable conditions; and c) detecting binding of EEC to each of the plurality of compounds, thereby identifying the compounds which specifically bind EEC.

Another technique for drug screening provides high throughput screening for compounds having suitable binding affinity to the EEC polypeptide and is described in detail in European Patent Application 84/03564, published on September 13, 1984, incorporated herein by reference. Briefly stated, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with EEC polypeptide and washed. Bound EEC polypeptide is then detected by methods well known in the art. Purified EEC can also be coated directly onto plates for use in the aforementioned drug screening techniques. In addition, non-neutralizing antibodies can be used to capture the peptide and immobilize it on the solid support.

This invention also contemplates the use of competitive drug screening assays in which neutralizing antibodies capable of binding EEC specifically compete with a test compound for binding to chemokine polypeptides or fragments thereof. In this manner, the antibodies can be used to detect the presence of any peptide which shares one or more antigenic determinants with EEC.

XII Rational Drug Design

The goal of rational drug design is to produce structural analogs of biologically active polypeptides of interest or of small molecules with which they interact, e.g., agonists, antagonists, or inhibitors. Any of these examples can be used to fashion drugs which are more active or stable forms of the polypeptide or which enhance or interfere with the function of a polypeptide in vivo (cf Hodgson J (1991) Bio/Technology 9:19-21, incorporated herein by reference).

In one approach, the three-dimensional structure of a protein of interest, or of a protein-inhibitor complex, is determined by x-ray crystallography, by computer modeling or, most typically, by a combination of the two approaches. Both the shape and charges of the polypeptide must be ascertained to elucidate the structure and to determine active site(s) of the molecule. Less often, useful information regarding the structure of a polypeptide may be gained by modeling based on the structure of homologous proteins. In both cases, relevant structural information is used to design analogous chemokine-like molecules or to identify efficient inhibitors. Useful examples of rational drug design include molecules which have improved activity or stability as shown by Braxton S and Wells JA (1992 Biochemistry 31:7796- 7801) or which act as inhibitors, agonists, or antagonists of native peptides as shown by Athauda SB et al (1993 J Biochem 113:742-746), incorporated herein by reference.

It is also possible to isolate a target-specific antibody, selected by functional assay, as described above, and then to solve its crystal structure. This approach, in principle, yields a pharmacore upon which subsequent drug design can be based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies (anti-ids) to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of the anti-ids would be expected to be an analog of the original receptor. In the present invention, an EEC anti-id antibody is used to identify and isolate peptides from banks of chemically or biologically produced peptides. The isolated peptides would then act as the

pharmacore.

By virtue of the present invention, sufficient amount of polypeptide may be made available to perform such analytical studies as X-ray crystallography. In addition, knowledge of the EEC amino acid sequence provided herein will provide guidance to those employing computer modeling techniques in place of or in addition to x-ray crystallography.

XIII Identification of EEC Receptors

Purified EEC are useful for characterization and purification of specific cell surface receptors and other binding molecules. Cells which respond to EEC by chemotaxis or other specific responses are likely to express a receptor for EEC. Radioactive labels are incorporated into EEC by various methods known in the art. A preferred embodiment is the labeling of primary amino groups in EEC with ^{125}I Bolton-Hunter reagent (Bolton, AE and Hunter, WM (1973) Biochem J 133: 529), which has been used to label other chemokines without concomitant loss of biological activity (Hebert CA et al (1991) J Biol Chem 266: 18989; McColl S et al (1993) J Immunol 150:4550-4555). Receptor-bearing cells are incubated with the labeled chemokine molecule. The cells are then washed to removed unbound chemokine, and receptor-bound labeled molecule is quantified. The data obtained using different concentrations of EEC are used to calculate values for the number and affinity of receptors.

Labeled EEC is also useful as a reagent for purification of its specific receptor. In one embodiment of affinity purification, the chemokine is covalently coupled to a chromatography column. Receptor-bearing cells are extracted, and the extract is passed over the column. The receptor binds to the column by virtue of its biological affinity for EEC. The receptor is recovered from the column and subjected to N-terminal protein sequencing. This amino acid sequence is then used to design degenerate oligonucleotide probes for cloning the receptor gene.

In an alternate method, mRNA is obtained from receptor-bearing cells and made into a cDNA library. The library is transfected into a population of cells, and those cells expressing the receptor are selected using fluorescently labeled EEC. The EEC specific receptor is identified by recovering and sequencing recombinant DNA from highly labeled cells.

In another alternate method, antibodies are raised against the surface of receptor- bearing cells, specifically monoclonal antibodies. The monoclonal antibodies are screened to identify those which inhibit the binding of labeled EEC. These monoclonal antibodies are then used in

affinity purification or expression cloning of the receptor.

Soluble receptors or other soluble binding molecules are identified in a similar manner. Labeled EEC is incubated with extracts or other appropriate materials derived from the eosinophil. After incubation, EEC complexes (which are larger than the size of purified the purified chemokine molecule) are identified by a sizing technique such as size exclusion chromatography or density gradient centrifugation and are purified by methods known in the art. The soluble receptors or binding protein(s) are subjected to N-terminal sequencing to obtain information sufficient for database identification, if the soluble protein is known, or for cloning, if the soluble protein is unknown.

All publications and patents mentioned in the above specification are herein incorporated by reference. The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Indeed, various modifications of the above described modes for carrying out the invention which are obvious to those skilled in the field of molecular biology or related fields are intended to be within the scope of the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: INCYTE PHARMACEUTICALS, INC.

(ii) TITLE OF INVENTION: NEW CHEMOKINE EXPRESSED IN EOSINOPHILS

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: INCYTE PHARMACEUTICALS, INC.
(B) STREET: 3174 Porter Drive
(C) CITY: Palo Alto
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 94304

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) PCT APPLICATION NUMBER: To Be Assigned
(B) FILING DATE: 12-APR-1996
(C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION SERIAL NO: US 08/421,144
(B) FILING DATE: 13-APR-1995

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Luther, Barbara J.
(B) REGISTRATION NUMBER: 33954
(C) REFERENCE/DOCKET NUMBER: PF-0031 PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 415-855-0555
(B) TELEFAX: 415-852-0195

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 411 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: EOSINOPHILS
(B) CLONE: 288236

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGAAGGTCT CCGTGGCTGC CCTCTCCTGC CTCATGCTTG TTACTGCCCT TGGATCCCAG 60

GCCCGGGTCA CAAAAGATGC AGAGACAGAG TTCATGATGT CAAAGCTTCC ATTGGAAAAT 120

CCAGTACTTC TGGACATGCT CTGGAGGAGA AAGATTGGTC CTCAGATGAC CCTTTCTCAT 180

GCTGCAGGAT TCCATGCTAC TAGTGCTGAC TGCTGCATCT CCTACACCCC ACGAAGCATC 240

CCGTGTTCAC TCCTGGAGAG TTACTTTGAA ACGAACAGCG AGTGCTCCAA GCCGGGTGTC 300

ATCTTCCTCA CCAAGAAGGG GCGACGTTTC TGTGCCAACC CCAGTGATAA GCAAGTTCAG 360

GTTTGCATGA GAATGCTGAA GCTGGACACA CGGATCAAGA CCAGGAAGAA T 411

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 137 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Eosinophils
(B) CLONE: 288236

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Lys Val Ser Val Ala Ala Leu Ser Cys Leu Met Leu Val Thr Ala
1 5 10 15

Leu Gly Ser Gln Ala Arg Val Thr Lys Asp Ala Glu Thr Glu Phe Met
20 25 30

Met Ser Lys Leu Pro Leu Glu Asn Pro Val Leu Leu Asp Met Leu Trp
35 40 45

Arg Arg Lys Ile Gly Pro Gln Met Thr Leu Ser His Ala Ala Gly Phe
50 55 60

His Ala Thr Ser Ala Asp Cys Cys Ile Ser Tyr Thr Pro Arg Ser Ile
65 70 75 80

Pro Cys Ser Leu Leu Glu Ser Tyr Phe Glu Thr Asn Ser Glu Cys Ser
85 90 95

Lys Pro Gly Val Ile Phe Leu Thr Lys Lys Gly Arg Arg Phe Cys Ala
100 105 110

Asn Pro Ser Asp Lys Gln Val Gln Val Cys Met Arg Met Leu Lys Leu
115 120 125

Asp Thr Arg Ile Lys Thr Arg Lys Asn
130 135

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 92 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Gln Val Ser Thr Ala Ala Leu Ala Val Leu Leu Cys Thr Met Ala
1 5 10 15

Leu Cys Asn Gln Phe Ser Ala Ser Leu Ala Ala Asp Thr Pro Thr Ala
20 25 30

Cys Cys Phe Ser Tyr Thr Ser Arg Gln Ile Pro Gln Asn Phe Ile Ala
35 40 45

Asp Tyr Phe Glu Thr Ser Ser Gln Cys Ser Lys Pro Gly Val Ile Phe
50 55 60

Leu Thr Lys Arg Ser Arg Gln Val Cys Ala Asp Pro Ser Glu Glu Trp
65 70 75 80

Val Gln Lys Tyr Val Ser Asp Leu Glu Leu Ser Ala
85 90

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 92 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Lys Leu Cys Val Thr Val Leu Ser Leu Leu Met Leu Val Ala Ala
1 5 10 15

Phe Cys Ser Pro Ala Leu Ser Ala Pro Met Gly Ser Asp Pro Pro Thr
20 25 30

Ala Cys Cys Phe Ser Tyr Thr Ala Arg Lys Leu Pro Arg Asn Phe Val
35 40 45

Val Asp Tyr Tyr Glu Thr Ser Ser Leu Cys Ser Gln Pro Ala Val Val
50 55 60

Phe Gln Thr Lys Arg Ser Lys Gln Val Cys Ala Asp Pro Ser Glu Ser
65 70 75 80

Trp Val Gln Glu Tyr Val Tyr Asp Leu Glu Leu Asn
85 90

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 99 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Lys Val Ser Ala Ala Leu Leu Cys Leu Leu Leu Ile Ala Ala Thr
1 5 10 15

Phe Ile Pro Gln Gly Leu Ala Gln Pro Asp Ala Ile Asn Ala Pro Val
20 25 30

Thr Cys Cys Tyr Asn Phe Thr Asn Arg Lys Ile Ser Val Gln Arg Leu
35 40 45

Ala Ser Tyr Arg Arg Ile Thr Ser Ser Lys Cys Pro Lys Glu Ala Val
50 55 60

Ile Phe Lys Thr Ile Val Ala Lys Glu Ile Cys Ala Asp Pro Lys Gln
65 70 75 80

Lys Trp Val Gln Asp Ser Met Asp His Leu Asp Lys Gln Thr Gln Thr
85 90 95

Pro Lys Thr

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 77 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ala Gln Pro Asp Ser Val Ser Ile Pro Ile Thr Cys Cys Phe Asn Val
1 5 10 15

Ile Asn Arg Lys Ile Pro Ile Gln Arg Leu Glu Ser Tyr Thr Arg Ile
20 25 30

Thr Asn Ile Gln Cys Pro Lys Glu Ala Val Ile Phe Lys Thr Lys Arg
35 40 45

Gly Lys Glu Val Cys Ala Asp Pro Lys Glu Arg Trp Val Arg Asp Ser
50 55 60

Met Lys His Leu Asp Gln Ile Phe Gln Asn Leu Lys Pro
65 70 75

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Trp Lys Pro Met Pro Ser Pro Ser Asn Met Lys Ala Ser Ala Ala
1 5 10 15

Leu Leu Cys Leu Leu Leu Thr Ala Ala Ala Phe Ser Pro Gln Gly Leu
20 25 30

Ala Gln Pro Val Gly Ile Asn Thr Ser Thr Thr Cys Cys Tyr Arg Phe
35 40 45

Ile Asn Lys Lys Ile Pro Lys Gln Arg Leu Glu Ser Tyr Arg Arg Thr
50 55 60

Thr Ser Ser His Cys Pro Arg Glu Ala Val Ile Phe Lys Thr Lys Leu
65 70 75 80

Asp Lys Glu Ile Cys Ala Asp Pro Thr Gln Lys Trp Val Gln Asp Phe
85 90 95

Met Lys His Leu Asp Lys Lys Thr Gln Thr Pro Lys Leu
100 105

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 91 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Lys Val Ser Ala Ala Arg Leu Ala Val Ile Leu Ile Ala Thr Ala
1 5 10 15

Leu Cys Ala Pro Ala Ser Ala Ser Pro Tyr Ser Ser Asp Thr Thr Pro
20 25 30

Cys Cys Phe Ala Tyr Ile Ala Arg Pro Leu Pro Arg Ala His Ile Lys
35 40 45

Glu Tyr Phe Tyr Thr Ser Gly Lys Cys Ser Asn Pro Ala Val Val Phe
50 55 60

Val Thr Arg Lys Asn Arg Gln Val Cys Ala Asn Pro Glu Lys Lys Trp
65 70 75 80

Val Arg Glu Tyr Ile Asn Ser Leu Glu Met Ser

85 90

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 86 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Lys Val Ser Val Ala Ala Leu Ser Val Leu Leu Leu Val Ala Ala
1 5 10 15

Leu Cys Asp Ala Gln Pro Thr Thr Cys Cys Phe Ser Tyr Thr Asn Arg
20 25 30

Lys Ile Pro Arg Gln Arg Leu Glu Ser Tyr Phe Glu Thr Ser Ser Gln
35 40 45

Cys Ser Lys Pro Ala Val Ile Phe Lys Thr Lys Arg Gly Lys Glu Val
50 55 60

Cys Ala Asp Pro Ser Glu Lys Trp Val Gln Asp Tyr Met Lys Leu Glu
65 70 75 80

Leu Asp Lys Gln Thr Lys
85

CLAIMS

1. A purified polynucleotide comprising a nucleic acid sequence encoding the polypeptide having the sequence as depicted in SEQ ID NO:2, or its complement.

2. The polynucleotide of Claim 1 wherein the nucleic acid sequence consists of SEQ ID NO:1.

3. An expression vector comprising the polynucleotide of Claim 2.

4. A host cell comprising the expression vector of Claim 3.

5. A nucleic acid probe comprising a non-conserved fragment of the polynucleotide of Claim 2.

6. The nucleic acid probe of Claim 5 comprising a nucleotide sequence encoding amino acid residues from amino acid 22 to 63, inclusive.

7. An antisense molecule comprising a polynucleotide sequence complementary to at least a portion of the polynucleotide of Claim 2.

8. A method for producing a polypeptide comprising the sequence as depicted in SEQ ID NO:2, said method comprising:

a) culturing the host cells of Claim 4 under conditions suitable for the expression of the polypeptide, and

b) recovering said polypeptide from the cell culture.

9. A purified Eosinophil Expressed Chemokine having the amino acid sequence as depicted in SEQ ID NO:2.

10. The purified Eosinophil Expressed Chemokine of Claim 9 having the N-terminal amino acid residue of residue 21, Alanine, of SEQ ID NO:2.

11. An antibody specific for the purified polypeptide of Claim 9.

12. A diagnostic composition for the detection of nucleic acid sequences

encoding Eosinophil Spleen Expressed Chemokine comprising the nucleic acid probe of Claim 6.

13. A diagnostic test for the detection of nucleic acid sequences encoding EEC in a biological sample, comprising the steps of:

a) combining the biological sample with a polynucleotide which comprises the nucleic acid sequence of SEQ ID NO:1, or a fragment thereof, under conditions suitable for the formation of a nucleic acid hybridization complex between the nucleic acid sequence of SEQ ID NO:1 and a complementary nucleic acid sequence in said sample,

b) detecting said hybridization complex, and

c) comparing the amount of said hybridization complex with a standard wherein the presence of an abnormal level of said hybridization complex correlates positively with a condition associated with inflammation.

14. A diagnostic test for the detection of nucleotide sequences encoding Eosinophil Spleen Expressed Chemokine in a biological sample, comprising the steps of:

a)combining the biological sample with polymerase chain reaction primers under conditions suitable for nucleic acid amplification, wherein said primers comprise fragments from non-conserved regions of the nucleotide sequence of SEQ ID NO:1;

b) detecting amplified nucleotide sequences; and

c) comparing the amount of amplified nucleotide sequences in said biological sample with a standard thereby determining whether the amount of said nucleotide sequence varies from said standard, wherein the presence of an abnormal level of said nucleotide sequence correlates positively with a condition associated with aberrant expression of EEC

15. A method of screening a plurality of compounds for specific binding affinity with the polypeptide of Claim 8 or any portion thereof, comprising the steps of:

a) providing a plurality of compounds;

b) combining Eosinophil Spleen Expressed Chemokine (EEC) with each of a plurality of compounds for a time sufficient to allow binding under suitable conditions; and

c) detecting binding of EEC to each of the plurality of compounds, thereby identifying the compounds which specifically bind EEC.

```
                9          18          27          36          45          54
5' ATG AAG GTC TCC GTG GCT GCC CTC TCC TGC CTC ATG CTT GTT ACT GCC CTT GGA
   Met Lys Val Ser Val Ala Ala Leu Ser Cys Leu Met Leu Val Thr Ala Leu Gly

               63          72          81          90          99         108
   TCC CAG GCC CGG GTC ACA AAA GAT GCA GAG ACA GAG TTC ATG ATG TCA AAG CTT
   Ser Gln Ala Arg Val Thr Lys Asp Ala Glu Thr Glu Phe Met Met Ser Lys Leu

              117         126         135         144         153         162
   CCA TTG GAA AAT CCA GTA CTT CTG GAC ATG CTC TGG AGG AGA AAG ATT GGT CCT
   Pro Leu Glu Asn Pro Val Leu Leu Asp Met Leu Trp Arg Arg Lys Ile Gly Pro

              171         180         189         198         207         216
   CAG ATG ACC CTT TCT CAT GCT GCA GGA TTC CAT GCT ACT AGT GCT GAC TGC TGC
   Gln Met Thr Leu Ser His Ala Ala Gly Phe His Ala Thr Ser Ala Asp Cys Cys

              225         234         243         252         261         270
   ATC TCC TAC ACC CCA CGA AGC ATC CCG TGT TCA CTC CTG GAG AGT TAC TTT GAA
   Ile Ser Tyr Thr Pro Arg Ser Ile Pro Cys Ser Leu Leu Glu Ser Tyr Phe Glu

              279         288         297         306         315         324
   ACG AAC AGC GAG TGC TCC AAG CCG GGT GTC ATC TTC CTC ACC AAG AAG GGG CGA
   Thr Asn Ser Glu Cys Ser Lys Pro Gly Val Ile Phe Leu Thr Lys Lys Gly Arg

              333         342         351         360         369         378
   CGT TTC TGT GCC AAC CCC AGT GAT AAG CAA GTT CAG GTT TGC ATG AGA ATG CTG
   Arg Phe Cys Ala Asn Pro Ser Asp Lys Gln Val Gln Val Cys Met Arg Met Leu

              387         396         405
   AAG CTG GAC ACA CGG ATC AAG ACC AGG AAG AAT  3'
   Lys Leu Asp Thr Arg Ile Lys Thr Arg Lys Asn
```

FIGURE 1

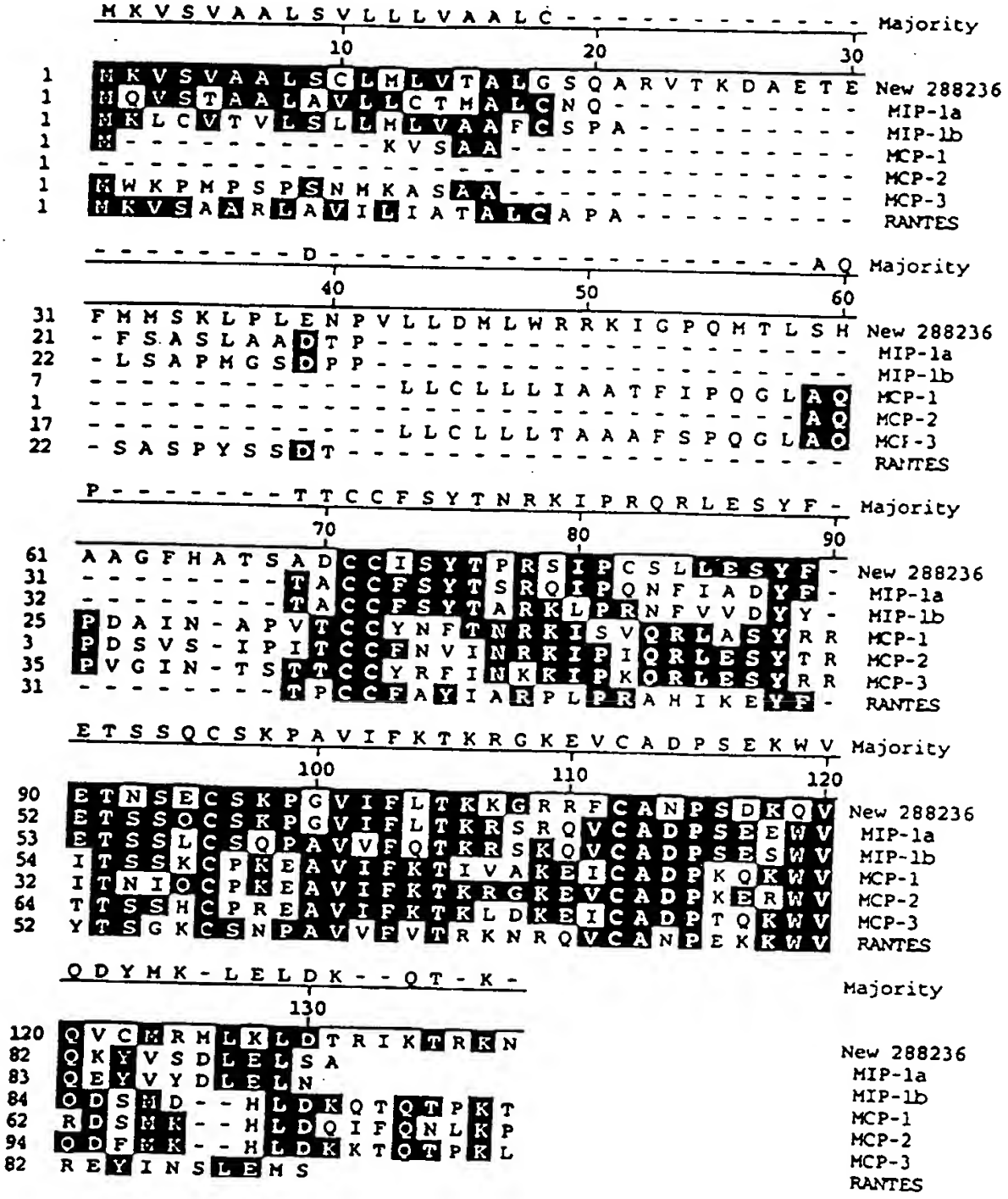

FIGURE 2

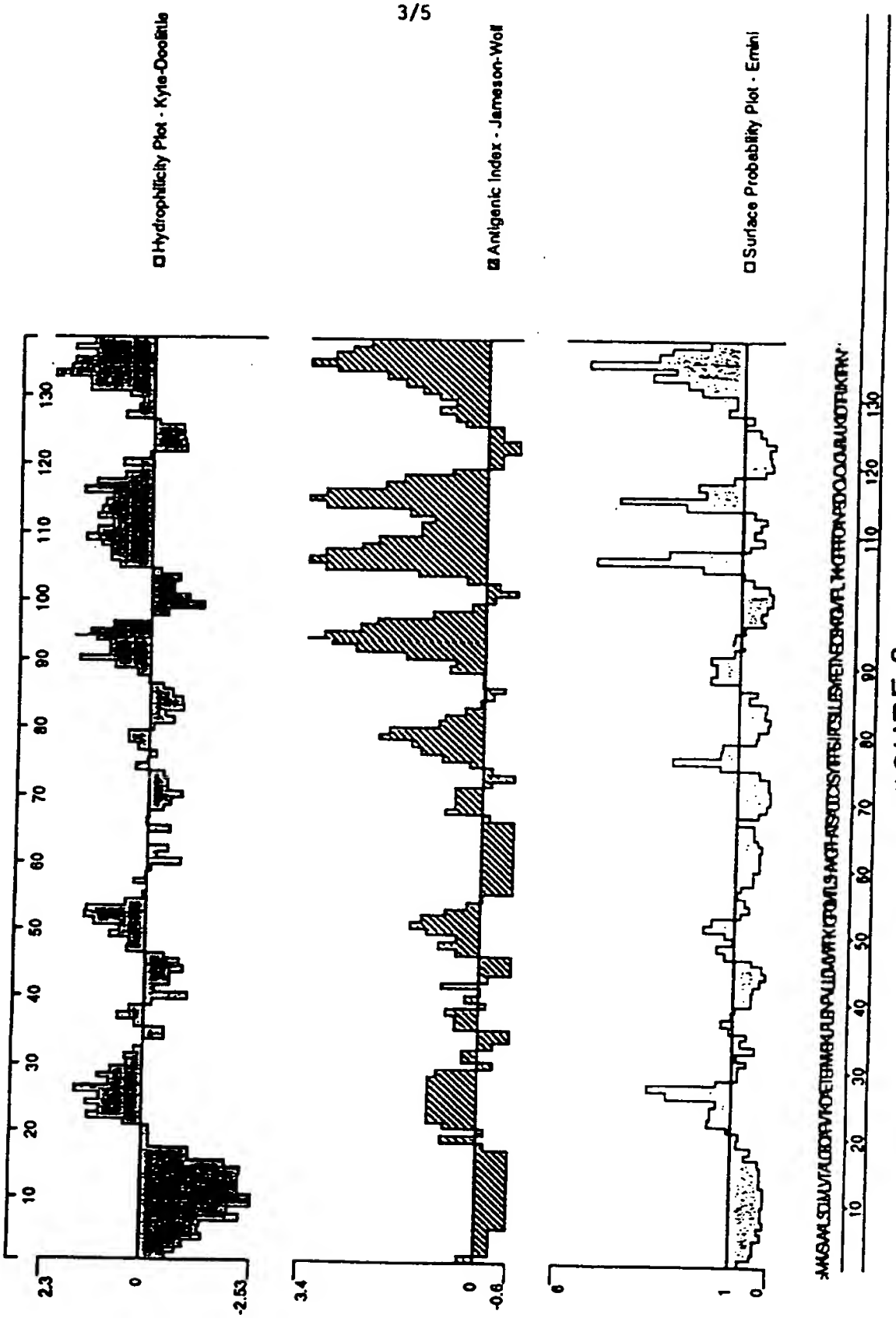
Hydrophilicity Plot - Kyte-Doolitle
Antigenic Index - Jameson-Wolf
Surface Probability Plot - Emini

FIGURE 3

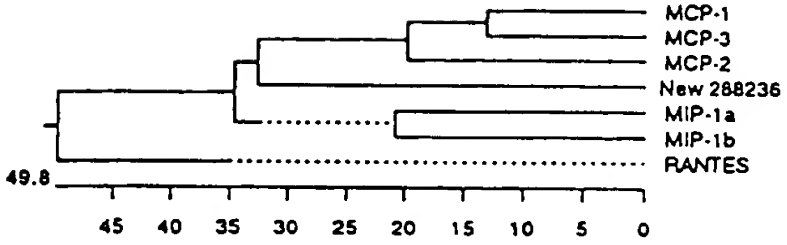
MCP-1
MCP-3
MCP-2
New 288236
MIP-1a
MIP-1b
RANTES
49.8
45 40 35 30 25 20 15 10 5 0

FIGURE 4

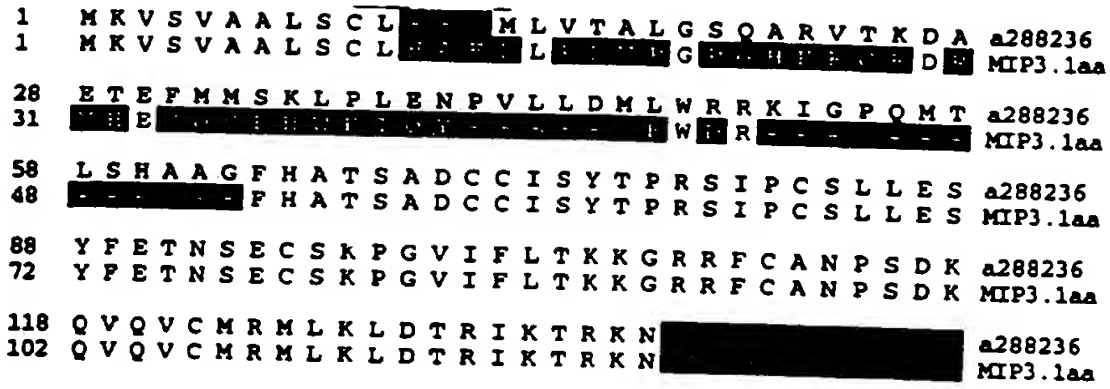
1 M K V S V A A L S C L M L V T A L G S Q A R V T K D A a288236
1 M K V S V A A L S C L L G D MIP3.1aa
28 E T E F M M S K L P L E N P V L L D M L W R R K I G P Q M T a288236
31 E W R MIP3.1aa
58 L S H A A G F H A T S A D C C I S Y T P R S I P C S L L E S a288236
48 F H A T S A D C C I S Y T P R S I P C S L L E S MIP3.1aa
88 Y F E T N S E C S K P G V I F L T K K G R R F C A N P S D K a288236
72 Y F E T N S E C S K P G V I F L T K K G R R F C A N P S D K MIP3.1aa
118 Q V Q V C M R M L K L D T R I K T R K N a288236
102 Q V Q V C M R M L K L D T R I K T R K N MIP3.1aa

FIGURE 5

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 96/05102

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/19 C07K14/52 C12Q1/68 C12N15/11 C07K16/24
G01N33/68 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MOLECULAR AND CELLULAR BIOLOGY, vol. 10, no. 7, July 1990, WASHINGTON US, pages 3646-3658, XP002005589 MITSUYOSHI NAKAO ET AL: "Structures of human genes coding for cytokine LD78 and their expression" ---	
P,A	WO,A,95 17092 (HUMAN GENOME SCIENCES) 29 June 1995 cited in the application ---	
A	WO,A,94 24285 (CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED) 27 October 1994 -----	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
13 June 1996	26.06.96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	**Authorized officer** Le Cornec, N

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INTERNATIONAL SEARCH REPORT

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Inte ..nal Application No
PCT/US 96/05102

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